Immunity, Vol. 21, 55–66, July, 2004, Copyright ©2004 by Cell Press

Identification of Probabilistic Transcriptional Switches in the *Ly49* Gene Cluster: A Eukaryotic Mechanism for Selective Gene Activation

Ali Saleh,[1,4] Gareth E. Davies,[3,4] Veronique Pascal,[1] Paul W. Wright,[3] Deborah L. Hodge,[1] Edward H. Cho,[2] Stephen J. Lockett,[2] Mehrnoosh Abshari,[3] and Stephen K. Anderson[3,*]

[1]Laboratory of Experimental Immunology
Center for Cancer Research
National Cancer Institute
[2]Image Analysis Laboratory
Science Applications International Corporation
[3]Basic Research Program
Science Applications International Corporation-Frederick
National Cancer Institute-Frederick
Frederick, Maryland 21702

Summary

Murine natural killer cells selectively express members of the Ly49 family of class I MHC receptors; however, the molecular mechanism controlling probabilistic expression of Ly49 proteins has not been defined. A pair of overlapping, divergent promoters discovered in the *Ly49g* gene functions as a molecular switch that can produce a forward transcript containing the coding region of the gene (on position) or a noncoding transcript in the opposite direction (off position), and this element maintains transcription in the chosen direction. Competition of C/EBP and TBP transcription factors for overlapping binding sites determines the relative strength of the competing promoters and the probability of transcription in a given direction. Similar elements precede all *Ly49* family members, and the relative strength of the forward promoter in each inhibitory *Ly49* gene correlates with the percentage of natural killer cells that express a given receptor, supporting a promoter competition model of selective gene activation.

Introduction

The ability of an organism to accurately sense a complex environment requires the development of a complex sensory system. Selective expression of sensory receptors allows for the detection of complex stimuli by generating subsets of sensory cells containing different combinations of receptors. Individual sensory cells are thus tuned to detect specific combinations of stimuli. Receptors for taste and smell discrimination represent large gene families, and individual receptors are selectively expressed on sensory cells to allow the identification of complex tastes and odors (Mombaerts, 2001; Montmayeur and Matsunami, 2002). Although monoallelic activation of gene expression has been described in several systems (Holländer et al., 1998; Bix and Locksley, 1998; Held and Kunz, 1998) and the assembly of transcriptional complexes is described as a probabilistic process (Hume, 2000), there has been no molecular explanation of the selective transcriptional activation of individual genes in a cluster of related receptor genes.

The Ly49 family of class I major histocompatibility (MHC) receptors expressed on murine natural killer (NK) cells represents a well-characterized system of selective gene expression (Anderson et al., 2001). NK cells are large granular lymphocytes that constitute an important component of the innate immune system and provide an early response to viral infection and tumor development (reviewed by Trinchieri, 1989). NK cell activity is controlled by receptors for class I MHC that inhibit killing of targets with normal expression of class I MHC molecules (the missing self hypothesis, Ljunggren and Karre, 1990). Human NK cells use the immunoglobulin-related killer cell inhibitory receptor (KIR) family of proteins to perform this function while mouse NK cells use the C-type lectin-related Ly49 proteins (Lanier et al., 1997). The *Ly49* gene cluster has been characterized in both C57BL/6 and 129/J mice, and there are at least 14 *Ly49* genes clustered in a head to tail array on mouse chromosome 6 (Wilhelm et al., 2002; Makrigiannis et al., 2002).

Each NK cell expresses a subset of the Ly49 class I MHC receptors, presumably to enable detection of potential targets that have lost expression of specific class I MHC proteins due to viral infection or malignant transformation. Individual Ly49 proteins are expressed on a consistent percentage of NK cells in a given mouse strain, and the probabilistic nature of *Ly49* gene activation was suggested by the observation that the proportion of NK cells expressing two Ly49 proteins is roughly equivalent to the product of the proportion of NK cells expressing each of the individual receptors (the product rule, Held et al., 1996). The analysis of several *Ly49* genes revealed that expression is predominately monoallelic and each allele is independently chosen for stable expression by a probabilistic mechanism (Held and Kunz, 1998). Single-cell RT-PCR analysis of *Ly49* mRNAs demonstrated that the selective expression of Ly49 proteins is controlled at the level of transcription (Kubota et al., 1999). The majority of NK cells transcribe from one to four different *Ly49* genes per cell, and an NK cell with five or more active *Ly49* genes is extremely rare. Detailed analyses of the *Ly49* promoters active in mature NK/NK-T cells have demonstrated the role of *cis*-acting elements and specific transcription factors in gene activation and cell-specific transcription (Held et al., 1999; Kubo et al., 1999; Gosselin et al., 2000; McQueen et al., 2001; Saleh et al., 2002).

The discovery of a novel *Ly49g* promoter (Pro1) upstream of the previously studied *Ly49* promoter (Pro2) suggested a possible role in the initiation of Ly49 expression since Pro1 was only active in immature NK cells (Saleh et al., 2002). The current study presents a detailed analysis of the Pro1 element, demonstrating bidirectional transcriptional activity due to the presence of overlapping divergent promoters. The direction of transcription from this element was studied in vivo by linking the cyan and yellow fluorescent protein genes to either

*Correspondence: andersn@ncifcrf.gov
[4]These authors contributed equally to this work.

side of the Pro1 element. Individual cells containing a single copy of the Pro1 element maintained transcription in either the forward or reverse direction, and the frequency of cells transcribing in a given direction correlated with the relative activity of the competing promoters. Dividing cells were capable of producing daughters with Pro1 transcription in opposite directions, demonstrating independent probabilistic choice of the direction of transcription by the new copy of the Pro1 element. These features are consistent with Pro1 functioning as a probabilistic molecular switch reminiscent of the λ phage right operator (Johnson et al., 1981). The λ right operator contains two competing divergent promoters that act as a programmed probabilistic switch governing the decision of the phage to enter the lytic or lysogenic phase of the λ life cycle. The probability of transcription in either the lytic or lysogenic direction is determined by the relative affinities of the competing promoters for RNA polymerase. The Pro1 element represents an example of a DNA-based eukaryotic decision–making element, and it provides a simple genetic solution to the problem of probabilistic gene activation.

Results

Pro1 Contains a Pair of Overlapping, Divergent Promoters

The detection of significant promoter activity from an inverted *Ly49g* Pro1 element led to further investigation of Pro1. A detailed analysis of the transcriptional activity of the *Ly49g* Pro1 region is shown in Figure 1. The 119 bp core promoter previously described (Saleh et al., 2002) exhibits nearly equivalent transcriptional activity in either orientation (12-fold reverse and 11-fold forward activity relative to control). Analysis of the core promoter sequence revealed the presence of a second TATA element (TATAAAT) 87 nucleotides upstream of the TATA element (TATAAAT) associated with the *Ly49g* Pro1 transcripts detected in immature NK cells (Saleh et al., 2002). Mutation of the 5′ TATA element abrogated reverse promoter activity (mutant 1), while mutation of the 3′ TATA eliminated forward transcriptional activity (mutant 12). The 5′ TATA element is on the opposite strand, indicating that an ATTTATA element is associated with leftward transcription. Several potential C/EBP sites, an Ets binding site, a GATA-3 element, and an NF-κB site were identified on the upper strand, while AML-1, NF-κB, and a single C/EBP site were found on the lower strand. The Ets site is overlapping an NF-κB site present on the opposite strand, and the reverse AML-1 site overlaps a second NF-κB site on the forward strand. Simultaneous disruption of the opposing Ets and NF-κB binding sites (mutant 3) resulted in the loss of activity in both directions, indicating an important role in the function of the Pro1 element. Mutation of the Ets-specific portion of this site also eliminated forward and reverse activity (mutant 2). However, selective mutation of the NF-κB-specific portion of this site abrogated reverse promoter activity and decreased forward activity (mutant 4), indicating that binding of NF-κB proteins to this site may preferentially induce Pro1 transcription in the reverse direction. Mutation of the NF-κB-specific half of the AML-1/NF-κB site decreased forward Pro1 transcription to a greater extent than reverse transcription (mutant 6), suggesting that NF-κB binding to this site may enhance forward transcription. Mutation of the AML-1 site resulted in a complete loss of transcriptional activity in both directions (mutant 5). There are three C/EBP sites surrounding the TATA associated with forward transcription, and disruption of the first (C/EBP1, mutant 8) or second (C/EBP2, mutant 9) site was able to significantly enhance the relative activity of the forward promoter. This suggests that C/EBP bound to either of these two C/EBP sites interferes with recruitment of the TATA binding protein (TBP) to the forward TATA element and results in decreased forward promoter activity in the wild-type Pro1 element. The decreased reverse promoter activity of mutants 8 and 9 suggests that C/EBP binding to these sites also enhances reverse transcription. Disruption of the C/EBP site that overlaps the forward TATA (C/EBP3, mutants 10 and 12) resulted in decreased reverse promoter activity, suggesting that binding of C/EBP to this site might also contribute to TBP recruitment to the reverse TATA element. Consistent with this hypothesis, a mutation of the forward TATA element that produced a C/EBP3 site more closely related to a consensus C/EBP binding site (Osada et al., 1996) significantly enhanced reverse promoter activity (mutant 11). Mutation of the C/EBP site overlapping the reverse TATA element (C/EBP-1, mutant 1) decreased the forward promoter activity, indicating that each TATA contains an overlapping element that enhances transcription from the opposing promoter. Simultaneous disruption of overlapping TATA and C/EBP elements at either end reduced forward and reverse promoter activity (mutants 1 and 12).

Switching Properties of the Bidirectional Promoter Complex

The competitive arrangement of the forward and reverse promoters in the Pro1 element suggested that it could function as a molecular binary switch with distinct probabilities of transcribing in either the forward or reverse direction as determined by the competition of transcription factors for the overlapping binding sites present in each promoter region. A two-color Pro1 reporter vector was designed to study the competition between the forward and reverse promoters in vivo. The destabilized yellow fluorescent protein (YFP) gene was linked to the forward promoter, and the destabilized cyan fluorescent protein (CFP) gene was linked to the reverse promoter (Figure 2A). Promoter fragments containing either the wild-type *Ly49g* Pro1 element or a mutant that demonstrated increased forward transcriptional activity (Figure 1, mutant 9) were cloned into the two-color vector, and the resulting constructs were used to isolate single-copy stable transfectants of the LNK cell line. Transfectants containing only a single copy of the vector were necessary to determine the switching properties of this element, since transfectants containing multiple copies of the vector would be expected to express both CFP and YFP, preventing the detection of switching events if the direction of transcription chosen by each element is independent of the state of the other copies. The independent behavior of the elements was suggested by the observation that clones containing multiple copies of

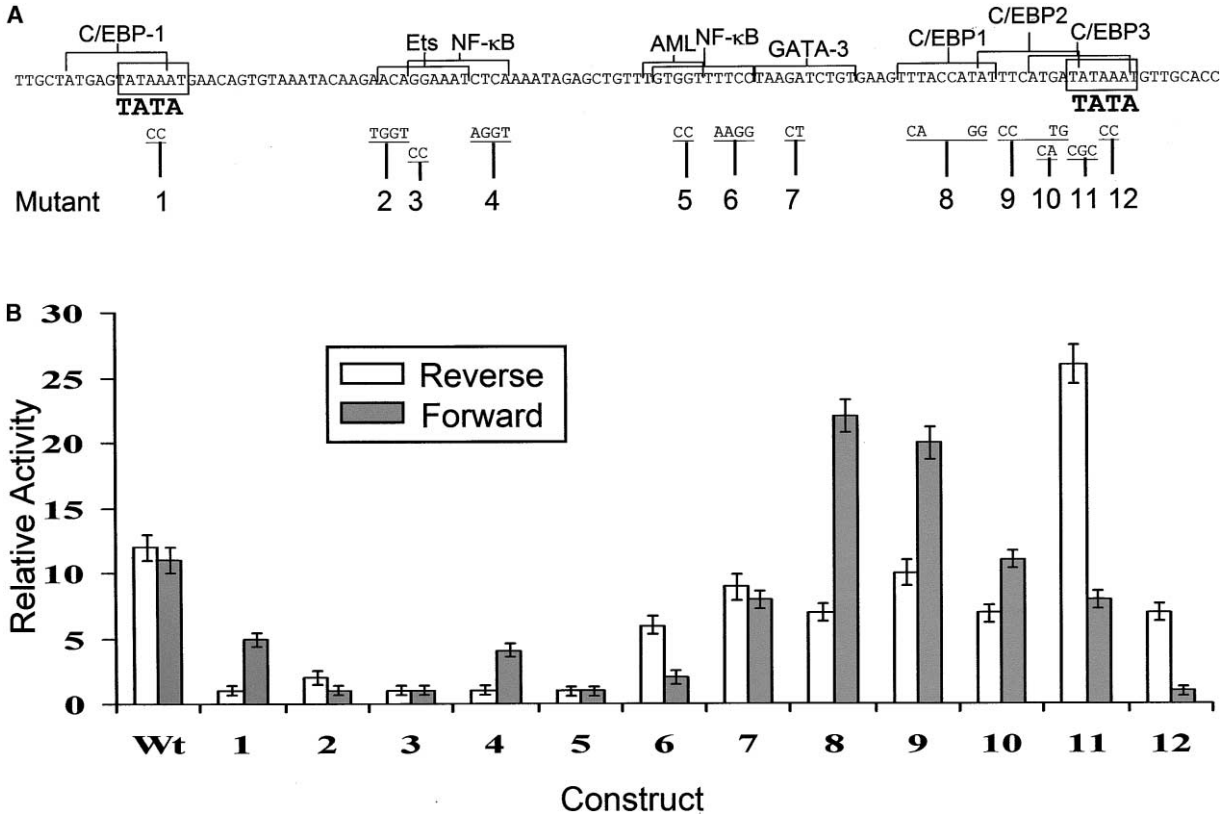

Figure 1. Characterization of Pro1 Bidirectional Transcription

(A) The nucleotide sequence of the wild-type *Ly49g* Pro1 element is shown, and potential transcription factor binding sites are indicated. Individual mutant constructs are numbered, and the modified nucleotides in each construct are shown below the wild-type sequence.

(B) The luciferase activity of pGL3 constructs containing the wild-type (Wt) *Ly49g* Pro1 region and mutants 1–12 cloned in either the forward (gray bar) or reverse (white bar) orientation is shown as fold increase of corrected light units relative to an empty pGL3 vector control.

the vector expressed both YFP and CFP protein (data not shown). To enrich for single-copy clones, G418-resistent cells were separated by fluorescence-activated cell sorting (FACS) to isolate cells expressing only YFP or CFP, followed by single-cell cloning. To ensure that switch behavior was not affected by the presence of the neomycin cassette, additional clones were generated with a construct containing only Pro1 and the divergent fluorescent protein genes (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/1/55/DC1). Clones were observed under a fluorescent microscope, and time-lapse images were acquired. An active forward promoter should produce yellow cells, and reverse promoter activity should result in cyan cells. Figure 2B demonstrates that single-cell clones containing the wild-type *Ly49g* Pro1 element produced populations containing approximately equal numbers of yellow and cyan cells, consistent with the balanced forward and reverse promoter activities observed in vitro (Figure 1, Wt construct). The exclusive expression of either CFP or YFP in individual cells was demonstrated by FACS analysis of cyan or yellow cells sorted from the population shown in Figure 2B. The majority of cyan cells had no detectable expression of YFP, and yellow cells did not have any CFP signal (Figure 2C). Cells transfected with the mutant *Ly49g* promoter lacking the C/EBP2 site (Figure 1, construct 9) that overlaps the forward TATA element produced populations expressing mainly YFP (forward transcript), confirming that disruption of this site significantly increases the probability of forward transcription. Time-lapse imaging of the LNK clone containing the wild-type *Ly49g* Pro1 element indicated that once a direction of transcription was chosen, it was maintained, and dividing cells could produce daughter cells that expressed different colors (Figure 2D). The parental cells that produced daughters of different colors expressed both CFP and YFP prior to cell division, indicating that the two copies of the Pro1 element produced as a result of DNA replication behaved independently. It is therefore unlikely that the direction of transcription chosen by this element is controlled by the cellular levels of relevant transcription factors, but rather by the probabilistic outcome of the competition at the overlapping promoters.

NF-κB p50 and AML-1 Interact with Pro1

To identify transcription factors potentially involved in the regulation of Pro1 activity in LNK cells, Western blotting was performed with antibodies reactive with candidate transcription factors, and EMSA analyses were performed with mutant and wild-type oligonucleotides spanning the predicted binding sites shown in Figure 1. The Ets-1/NF-κB site adjacent to the reverse TATA element was critical for transcription in either direction

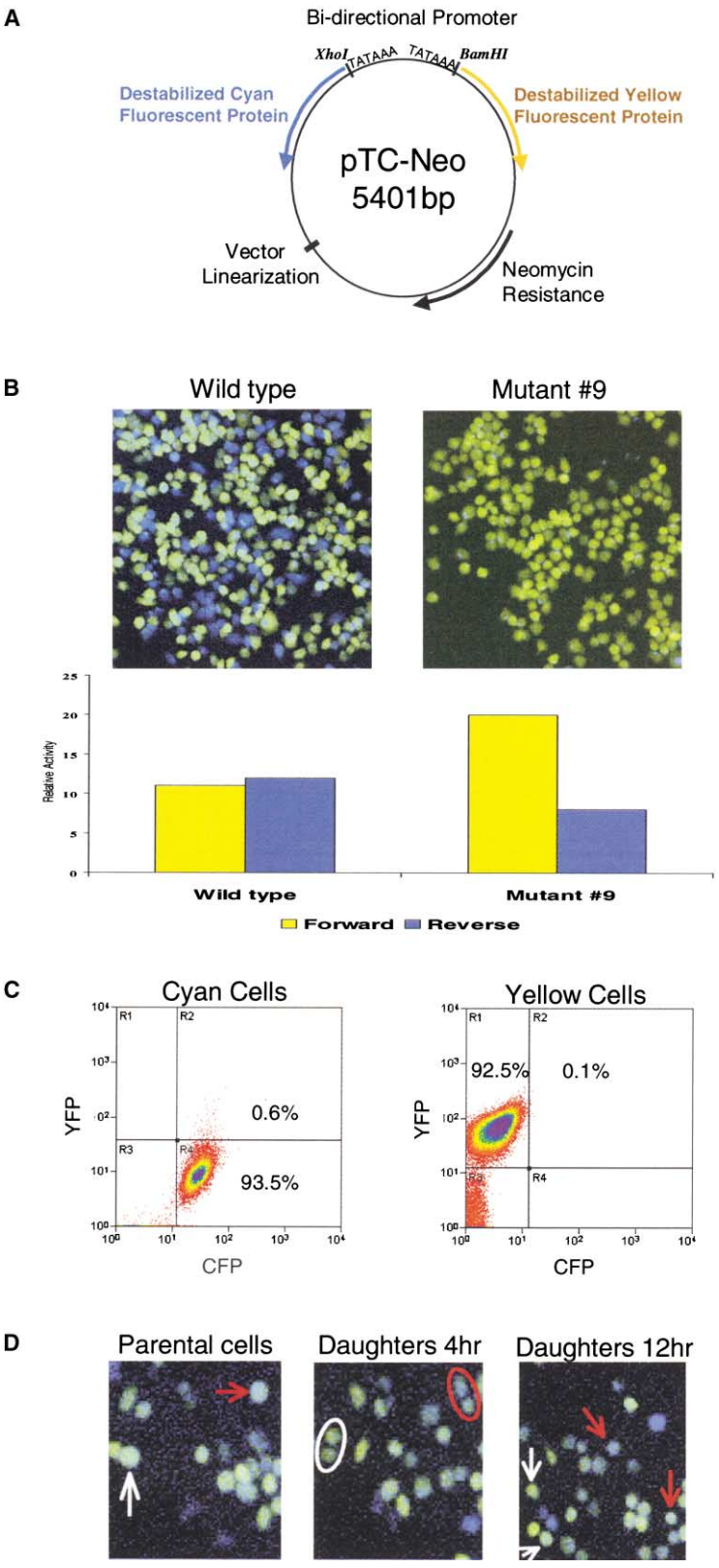

Figure 2. Switching Properties of the *Ly49g* Pro1 Element

(A) Two-color vector pTC-Neo. Wild-type or mutant 9 Pro1 constructs shown in Figure 1 were inserted such that YFP is expressed from a forward transcript and CFP is expressed from a reverse transcript.

(B) Selective expression of either CFP or YFP from the *Ly49g* Pro1 two-color construct. Confocal images of single-cell clones of LNK cells transfected with two-color vectors containing the wild-type *Ly49g* Pro1 (left panel) or the mutant lacking a functional C/EBP2 site (mutant 9) with dominant forward activity (right panel) are shown. A graphic representation of the relative forward and reverse promoter activity determined for each construct is shown under the corresponding confocal image.

(C) Mutually exclusive expression of either CFP or YFP from the *Ly49g* Pro1 two-color construct. CFP- or YFP-expressing cells were sorted from the *Ly49g* population shown in Figure 2B. FACS analysis of the sorted cells is shown. The majority of CFP-sorted cells had no detectable expression of YFP, and YFP-sorted cells did not have any CFP signal. A small percentage of CFP and YFP double-positive cells was detected in each population.

(D) Independent choice of transcriptional direction by the new copy of Pro1 generated by DNA replication. Selected images from a 16 hr time course study of the wild-type *Ly49g* Pro1 transfectant are shown. Images were collected every 5 min over a 16 hr period. The color fate of daughter cells produced by two cells that divided 4 hr into the time course is shown. The arrows in the first panel indicate the two cells just before division. The ovals in the second panel indicate the daughter cells at 4 hr after division. The arrows in the third panel indicate the daughter cells 12 hr after division. Red symbols indicate the parental and daughter cells that assumed different "color fates."

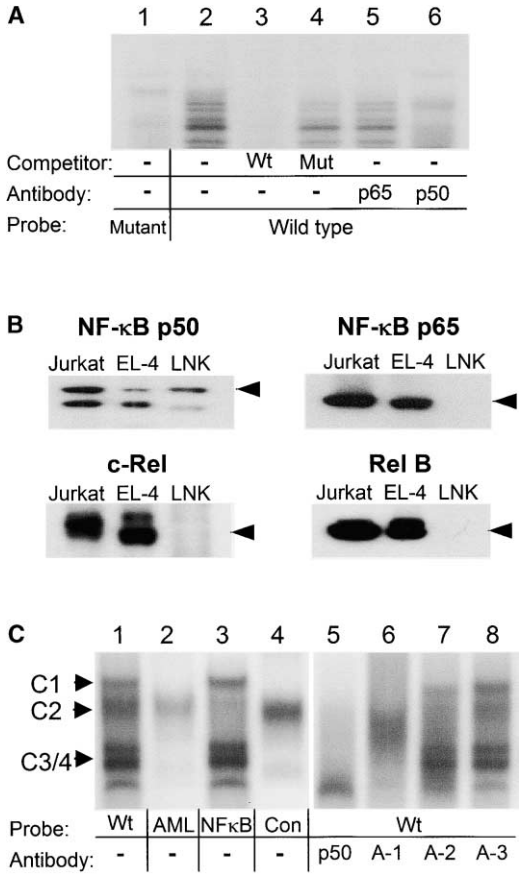

Figure 3. Transcription Factor Binding to the Central Region of the Pro1 Element

(A) The Ets/NF-κB site produces NF-κB containing complexes with LNK nuclear lysates. The wild-type Ets/NF-κB probe is described in the Experimental Procedures. EMSA was performed with oligonucleotides corresponding to wild-type (lanes 2–6) or the construct 3 mutation shown in Figure 1 (lane 1). A 50-fold excess of unlabeled competitor wild-type (Wt) or mutant (Mut) oligonucleotides or 1 μl of NF-κB p50 (p50) or p65 (p65) antibody was added to the LNK nuclear lysates 30 min prior to the addition of the labeled probe.

(B) LNK cells do not express NF-κB p65. Each panel represents a Western blot performed on Jurkat, EL4, and LNK lysates using antibodies specific for the NF-κB family members indicated. Arrows indicate the expected molecular weight of the NF-κB protein detected.

(C) The AML/NF-κB site binds both AML1 and NF-κB proteins in LNK nuclear lysates. EMSA was performed with oligonucleotide probes containing the AML/NF-κB site. The wild-type probe and consensus NF-κB probe (Con) are described in the Experimental Procedures. The AML-specific (-AML) and NF-κB-specific (-NF-κB) mutations correspond to those present in constructs 5 and 6 shown in Figure 1. Arrows labeled C1, C2, and C3/4 indicate the major complexes observed. Complexes were inhibited with antibodies reactive with AML-1 (A-1), AML-2 (A-2), AML-3 (A-3), or NF-κB p50 (p50).

(Figure 1, mutant 3), and an oligonucleotide containing this site produced DNA-protein complexes in LNK nuclear lysates that were inhibited by antibody reactive with the NF-κB p50 protein (Figure 3A, lane 6), suggesting that this site is preferentially bound by p50 dimers in LNK cells since NF-κB p65, c-Rel, and Rel B were not detected in LNK cells (Figure 3B). Antibodies against known Ets family members had no effect on complex formation in LNK cells; however, this element specifically bound Elf and MEF proteins in EL4 nuclear lysates (data not shown). Mutational analysis of the AML-1/NF-κB site adjacent to the forward TATA element indicated that the predicted AML-1 binding half of this site was essential for Pro1 transcriptional activity (Figure 1, mutant 5). A probe containing this element produced several specific complexes with LNK extracts (Figure 3C, lane 1). An anti-AML-1 antibody disrupted the complexes (Figure 3C, lane 6), whereas anti-AML-2 had a slight effect and anti-AML-3 had no effect on complex formation (Figure 3C, lanes 7 and 8). The higher molecular weight complex (C1) in LNK extracts was disrupted by the addition of either anti-p50 or anti-AML-1 antibodies, indicating the involvement of both of these factors in the formation of this complex. Mutant oligonucleotides lacking the AML-1 binding site (mutant 5 in Figure 1) resulted in a single complex containing NF-κB p50 protein (C2, lane 2), and elimination of the NF-κB-specific half of this site (mutant 6 in Figure 1) removed the NF-κB-specific complex (compare C2 in lanes 1 and 4 with lane 3), but not the higher molecular weight C1 complex, suggesting that this complex could result from direct interaction of AML-1 and p50 since anti-p50 also blocked formation of the C1 complex in this mutant (lane 5). Furthermore, mutation of the AML-1 site (TGTGGT; Meyers et al., 1993) to the consensus p50 binding sequence (GGGA; Chen et al., 1998) resulted in the loss of the major complexes, leaving only a complex containing p50 (C2, lane 4), indicating that AML-1 binding is required to generate complexes C1 and C3/4.

Cooperative Binding of C/EBP-δ, NF-κB p50, and TBP to Pro1

Western blotting of LNK lysates indicated that C/EBPγ and C/EBPδ were the only C/EBP family members expressed in this cell line (Figure 4A). The C/EBP site overlapping the left TATA element (Figure 1, C/EBP-1) was analyzed in conjunction with the adjacent NF-κB site to determine the role of these factors in recruitment of TBP (Figure 4B). An oligonucleotide probe containing this region produced a single large complex with LNK nuclear extracts (Figure 4B, lane 2). Mutation of the NF-κB site (corresponding to Figure 1, mutant 3) prevented complex formation (lane 1). Mutant oligonucleotides with specific disruption of the C/EBP and TATA sites (Figure 1, mutant 1) also failed to produce the TBP complex (data not shown). Depletion of C/EBPδ or TBP inhibited complex formation (Figure 4B, lanes 4 and 5), indicating cooperation of these transcription factors in the formation of a ternary complex. EMSA was performed with recombinant C/EBPδ, NF-κB p50, and TBP proteins to provide direct evidence for cooperative binding (Figure 4C). As expected, C/EBPδ and TBP together produced a strong complex whereas TBP alone only demonstrated weak binding, and there was no detectable C/EBPδ binding (Figure 4C, compare lanes 2, 3, and 5). NF-κB p50 and TBP proteins were also able to bind in a cooperative fashion (compare lanes 1, 3, and 6). To demonstrate that disruption of the TATA element (Figure 1, mutant 1) affected TBP binding, EMSA analysis with purified TBP protein was performed with this mutant.

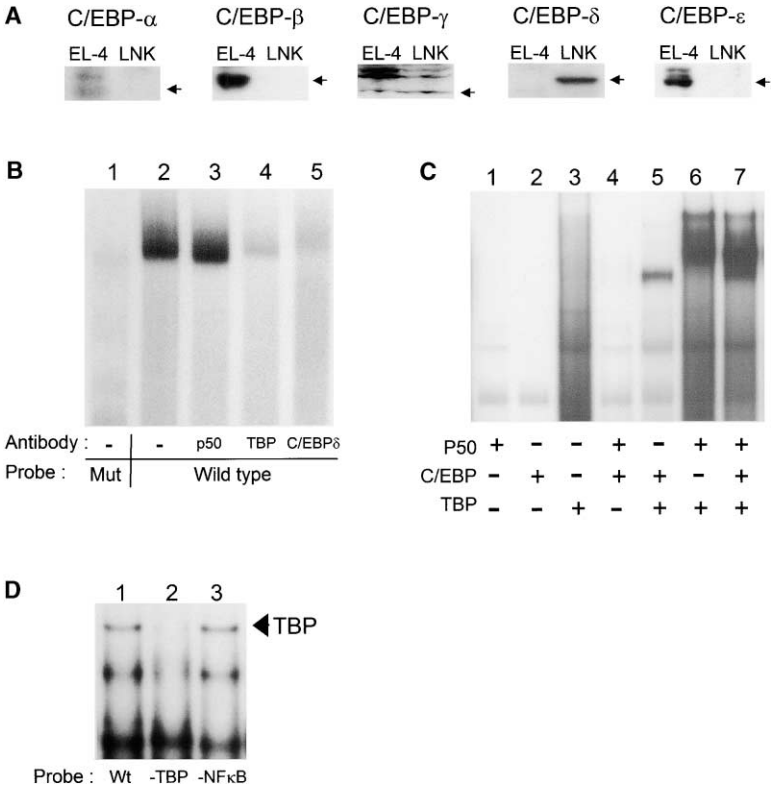

Figure 4. C/EBP-δ, NF-κB p50, and TBP Interact with the Reverse TATA Element and Flanking NF-κB Site

(A) LNK cells express C/EBPγ and C/EBPδ. Each panel represents a Western blot performed on protein lysates from EL4 or LNK cells with an antibody reactive with the C/EBP family member indicated. Arrows indicate the expected molecular weight of the C/EBP family member detected.

(B) Recruitment of TBP by C/EBP-1 and Ets/NF-κB sites requires C/EBPδ. EMSA was performed with oligonucleotide probes containing the region from the reverse TATA-C/EBP-1 site to the NF-κB site (Rev-TATA+NF-κB) as described in the Experimental Procedures. One microliter of antisera reactive with NF-κB p50 (p50), TATA binding protein (TBP), or C/EBPδ was added to LNK nuclear lysates to inhibit complex formation.

(C) Cooperative binding of TBP-C/EBP and TBP-NF-κB p50. EMSA was performed using the wild-type probe and 100 ng of recombinant NF-κB p50 (p50), C/EBPδ (C/EBP), or TBP protein in the combinations indicated.

(D) TBP binding is lost in the reverse TATA mutant. One hundred nanograms of TBP protein was added to probe corresponding to the wild-type probe (Wt) used in (B) and (C), or probes containing mutations corresponding to mutant 1 (-TBP), or mutant 3 (-NF-κB) in Figure 1.

As shown in Figure 4D (lane 2), disruption of the TATA element significantly reduced TBP binding. Antisera reactive with C/EBPγ, C/EBPδ, or NF-κB p50 were able to immunoprecipitate the Pro1 region in a chromatin immunoprecipitation assay, whereas C/EBPε and NF-κB p65 did not produce a significant signal (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/21/1/55/DC1), supporting an in vivo role for the transcription factors found to associate with Pro1 in vitro.

Competition among C/EBP Sites at the Forward Pro-1 TATA Element

Functional analysis of the contribution of individual C/EBP sites to Pro-1 transcription indicated that mutation of the C/EBP site overlapping the left TATA element (C/EBP-1) decreased forward transcriptional activity (Figure 1, mutant 1); mutation of either of the first two C/EBP sites flanking the right TATA element (C/EBP1 and C/EBP2) increased forward transcription (Figure 1, mutants 8 and 9), and mutation of the C/EBP3 site reduced reverse transcription (Figure 1, mutants 10 and 12). EMSA analysis was performed in order to determine which C/EBP family members were binding to the C/EBP sites adjacent to the forward TATA and the effect of site occupancy on TBP recruitment. Site 1 mutants demonstrated that formation of the TBP-containing complex is associated with a functional C/EBP1 binding site. Oligonucleotides containing an intact site 1 formed a high molecular weight complex (C1; lanes 1, 4, and 7 in Figure 5B), and this complex was inhibited by the addition of anti-TBP antibody (lanes 3, 6, and 9 in Figure 5B). If site 1 is mutated so that it no longer resembles a C/EBP site, the TBP-containing complex is eliminated (lane 10). These results demonstrate that binding of C/EBP-δ to site 1 is able to recruit TBP. Mutation of site 2 demonstrated that C/EBP-γ binds preferentially to this site. Oligonucleotides containing an intact site 2 were able to generate supershifted complexes containing C/EBP-γ (lanes 2, 5, and 11), but a site 2 mutant no longer produced the C/EBP-γ supershift complex (lane 8), and this enhanced the formation of the C/EBPδ-TBP complex (lanes 7 and 8). In addition, anti-C/EBPγ antibody increased the intensity of the C/EBPδ-TBP complex (lane 2). This is in agreement with the functional assays demonstrating that mutation of site 2 enhances forward transcriptional activity (Figure 1, mutant 9). C/EBP-γ lacks a *trans*-activation domain (Cooper et al., 1995); therefore, preferential binding of C/EBP-γ to this site is consistent with the role of site 2 in the inhibition of forward transcription. Elimination of site 3 (mutant 10) did not have a significant effect on the formation of the C/EBPδ-TBP complex (compare lanes 1 and 4), suggesting that site 2 has a dominant blocking effect on recruitment of TBP. A direct analysis of the binding of recombinant C/EBPγ and C/EBPδ to this region demonstrated that C/EBPγ-δ heterodimers were the predominant complex formed (Figure 5C, lanes 2, 4, 9, 11, and 13) and C/EBPδ alone did not show significant binding, indicating that C/EBP sites 1–3 have a low affinity for C/EBPδ as observed for the C/EBP-1 site (Figure 4C, lane 2, and data not shown). Homodimers of C/EBPδ were only detected in mutant 10 that specifically disrupted the C/EBP3 site but generated a stronger C/EBP binding consensus sequence at site 2 (Figure 5C, lane 8). Specific mutation of site 2 reduced C/EBP binding (Figure 5C, lane 6), suggesting

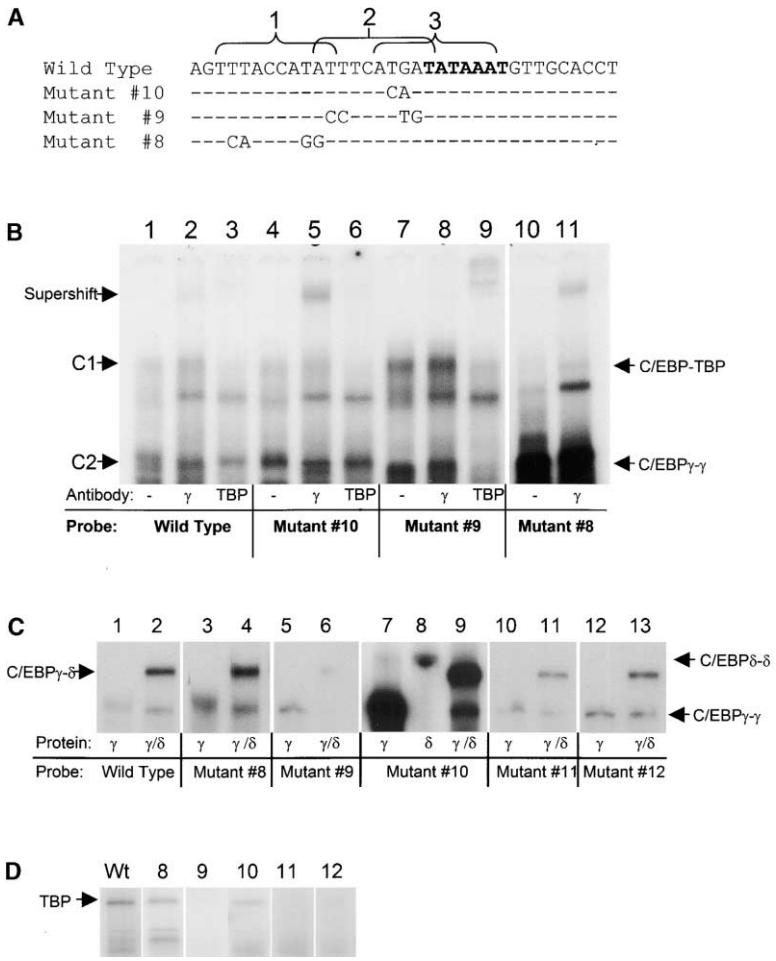

Figure 5. Effect of C/EBP Sites on the Recruitment of TBP to the Forward TATA

(A) The sequence of the double-stranded oligonucleotides used for EMSA analyses are shown. Only nucleotide differences are shown for the mutant oligonucleotides.

(B) EMSA analyses of the right C/EBP-TATA cluster. The oligonucleotides shown in (A) were used for an EMSA analysis with LNK nuclear extracts. Polyclonal rabbit antisera specific for either C/EBP-γ or TBP were preincubated with the extracts to block complex formation. The positions of C/EBPγ homodimers (C/EBPγ-γ), C/EBPδ-TBP complexes (C/EBP-TBP), or supershift complexes are indicated by arrows.

(C) Cooperative binding of C/EBPδ and C/EBPγ to Pro1. One hundred nanograms of recombinant C/EBPγ alone (γ) or in combination with C/EBPδ (γ/δ) was incubated with oligonucleotides corresponding to the wild-type probe and mutants 8–10 shown in (A) as well as the mutations present in constructs 11 and 12 in Figure 1. The positions of complexes containing C/EBPγ homodimers (C/EBPγ-γ), C/EBPγ-C/EBPδ heterodimers (C/EBPγ-δ), and a single C/EBPδ homodimer (C/EBPδ-δ) are indicated by arrows.

(D) TBP binding of C/EBP mutants. One hundred nanograms of recombinant TBP protein was added to oligonucleotide probes 8–12 as described above. The position of the TBP complex is indicated by the arrow.

that the increased forward activity of this mutant is in fact due to reduced competition from C/EBP bound adjacent to the forward TATA element, and TBP is recruited to the forward TATA element by C/EBP bound to the reverse TATA region. Figure 5D demonstrates that mutants with alterations in the TATA element (constructs 11 and 12) eliminated TBP binding, and the C/EBP mutations present in constructs 8 and 10 retained TBP binding. Surprisingly, construct 9 did not show TBP binding in this assay; however, a TBP-C/EBP complex could be formed by this mutant (lane 7, Figure 5B), and this construct demonstrated a strong forward promoter activity (Figure 1), indicating that C/EBP recruitment is able to compensate for the weaker TATA element.

Pro1 Forward Transcriptional Activity Correlates with Subset Expression

If the transcriptional switch is important for *Ly49* gene activation, the tendency of Pro1 to transcribe in the forward or reverse direction should correlate with the percentage of NK cells expressing a given Ly49. Each *Ly49* gene has a characteristic probability of activation, as evidenced by the significant differences in the percentage of NK cells expressing individual receptors. For example, the *Ly49c*, *i*, and *j* genes are highly homologous; however, the levels of expression of these receptors on C57BL/6 NK cells are 50%, 35%, and 5%, respectively (Kubota et al., 1999). Figure 6A shows a comparison of the core promoter regions of the *Ly49c, e*, *g*, *i*, and *j* genes. It is interesting to note that although the *Ly49c* and *i* genes have a TATA-related element slightly downstream of the *Ly49g* TATA, there is an additional C/EBP binding site that overlaps this element, conserving the competition between TBP and C/EBP at the forward TATA element. Figure 6B shows the relative forward and reverse promoter activities of the core Pro1 elements, and the relative strength of the forward promoters of *Ly49c*, *i, j*, and *g* correlated well with the size of the NK cell subset expressing each receptor. No forward promoter activity was detected from *Ly49e* Pro1, which may be due to the lack of a strong TATA element at the 3′ end of this promoter. However, forward Pro1 transcripts of *Ly49e* were cloned from fetal thymus by RT-PCR (Saleh et al., 2002), confirming that there is an active forward promoter in vivo. The LNK cell line was isolated from adult liver (Tsutsui et al., 1996), so the absence of forward *Ly49e* Pro1 activity in this line supports the role of this element in transcriptional activation, since Ly49E is expressed in fetal thymus (Van Beneden et al., 2001; Fraser et al., 2002), and *Ly49e* transcripts were not detected in adult NK cells (Kubota et al., 1999). Although *Ly49a* Pro1 forward transcripts were detected in vivo (Saleh et al., 2002), constructs containing the *Ly49a* Pro1 element displayed a domi-

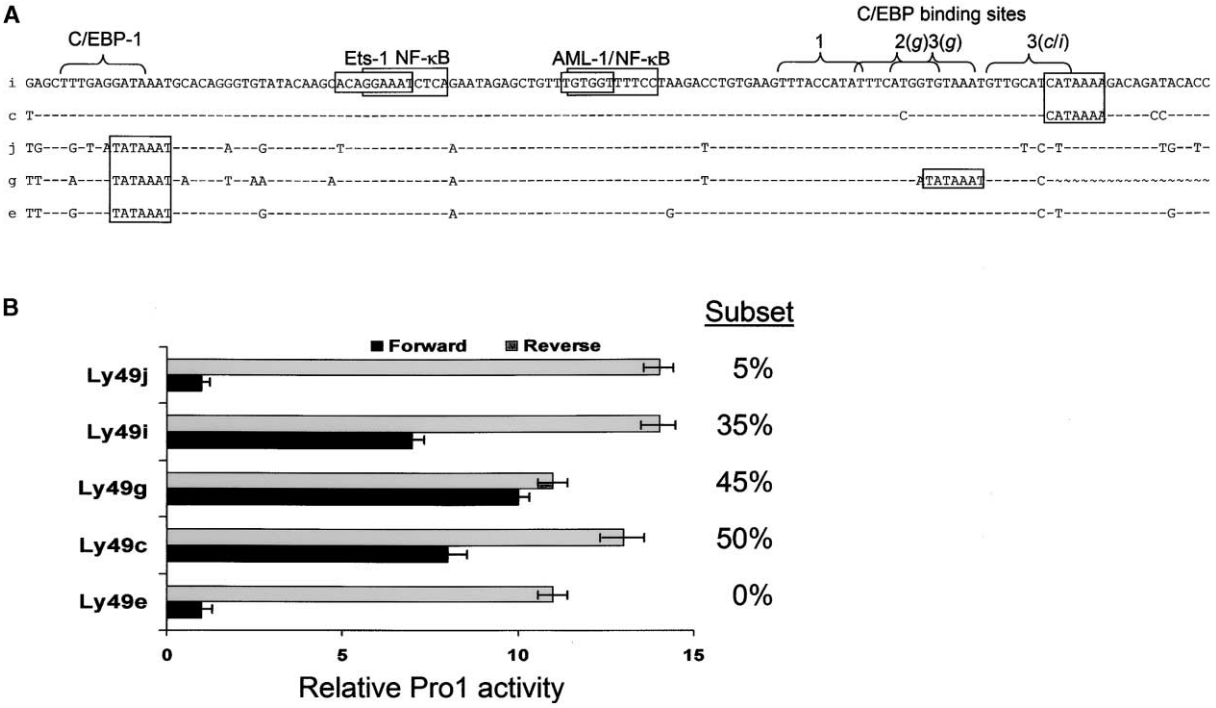

Figure 6. *Ly49* Genes Contain Pro1 Elements with Differing Forward and Reverse Promoter Strengths

(A) Comparison of the *Ly49* Pro1 elements. The sequence of the *Ly49i* Pro1 element is shown, and only differences are shown for the other *Ly49* genes. TATA elements are boxed, and brackets indicate C/EBP sites.

(B) The relative activity of core Pro1 elements of several *Ly49* genes. The activity of the Pro1 elements shown in (A) is shown as fold increase relative to a pGL3 control. Black bars indicate forward activity; gray bars indicate reverse activity. The observed subset expression of each Ly49 in adult spleen NK cells (Kubota et al., 1999) is shown for comparison with the relative forward activity of each promoter.

nant reverse activity similar to *Ly49e* (data not shown), which is consistent with *Ly49a* activation occurring at a developmental stage that is distinct from that of *Ly49g, c*, and *i* (Williams et al., 2000). Furthermore, attempts to demonstrate forward Pro1 activity from the activating *Ly49d* or *h* have been unsuccessful, raising the possibility that different elements may be involved in the control of these genes.

Discussion

The Pro1 Element Is a Probabilistic Switch

The series of experiments illustrated in Figure 2 demonstrates an important property of the Pro1 element: once a direction of transcription is chosen, the promoter maintains transcription in the same direction. This suggests that there is not a continual dynamic competition occurring, and transcription factors remain bound to the DNA between cycles of transcription. This observation is compatible with the bidirectional promoter complex acting as a switch with a predetermined probability of choosing either an on or off state for the locus. Since dividing cells were observed to produce daughter cells with different colors (Figure 2D), the new copy of the two-color Pro1 construct produced as a result of DNA replication behaves independently and chooses its "color fate" based on the probability of forward or reverse transcriptional complex assembly. The probability of forward or reverse transcription is programmed by the relative strength of the TATA and C/EBP elements of the competing divergent promoters. In the case of the *Ly49g* element, there is balanced forward and reverse promoter strength that results in the relatively equal probability of YFP or CFP production from the two-color Pro1 construct, and therefore clones containing this construct produced populations with approximately equal levels of cyan or yellow cells. A mutant of the *Ly49g* element that enhanced the relative activity of the forward promoter (mutant 9) produced clones that preferentially expressed the YFP protein, indicating that the relative strength of the opposing promoters in a Pro1 element can determine the percentage of cells expressing a particular protein.

The stability of the forward and reverse transcriptional states of this probabilistic switch suggests that once a given array of AML, NF-κB, C/EBP, and TBP proteins bind to this element, they lock on to the DNA. There is precedent for stable binding of TBP to DNA, since TBP has been shown to remain bound to DNA even in transcriptionally silent chromatin during mitosis (Christova and Oelgeschlager, 2001; Chen et al., 2002). NF-κB has been shown to have a very high affinity for DNA, suggesting that this complex may also be extremely stable (Thanos and Maniatis, 1992). The ability of the Pro1 bidirectional element to maintain transcription in either the forward or reverse direction suggests that C/EBP dimers may also form a tight association with DNA since C/EBP would have to be continuously bound to the

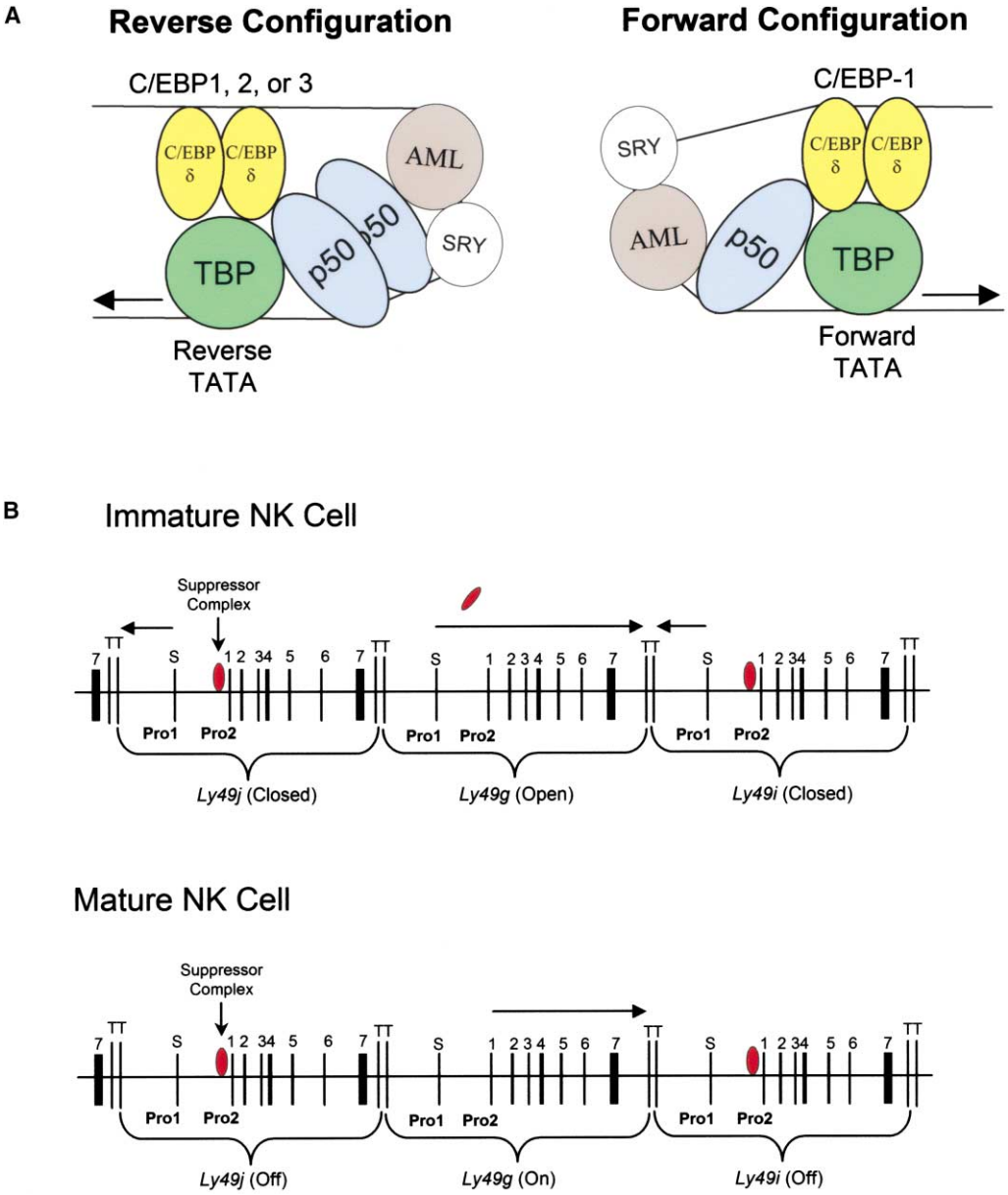

Figure 7. Models of Stochastic *Ly49* Gene Activation

(A) Mutually exclusive states of the Pro1 element. The proposed assembly of transcription factors on the Pro1 element required for transcription in the forward (forward configuration) or reverse (reverse configuration) is shown. The position of relevant C/EBP or TATA elements for each configuration is shown, and proposed transcription factors are labeled.

(B) Proposed Pro1 function in *Ly49* gene activation. A schematic based on the known sequence of the C57BL/6 *Ly49j, g*, and *i* genes is shown (Wilhelm et al., 2002). Arrows in the upper panel indicate an example of a set of theoretical Pro1 transcriptional "decisions" in immature NK cells. Forward transcripts are predicted to interfere with the formation of a suppressor complex at Pro2. The positions of the Pro1 and Pro2 promoters in each *Ly49* gene are indicated. The lower panel indicates the selective *Ly49* gene activation in mature NK cells predicted by the Pro1 activities shown in immature NK cells. Since only the *Ly49g* gene was derepressed by a forward Pro1 transcript, it is the only gene responsive to Pro2-specific transcription factors present in mature NK cells. The Pro1 promoter is not active in mature NK cells (Saleh et al., 2002).

opposing TATA element to prevent spontaneous switching of the direction of transcription.

The Predicted Structural Configurations of Forward and Reverse Pro1 Transcriptional Complexes Are Mutually Exclusive

The functional and EMSA analyses suggest the following model to account for the probabilistic nature of the choice of transcriptional direction made by the Pro-1 switch. The TATA and C/EBP sites required for transcription in one direction are in competition with the TATA and C/EBP sites necessary for transcription in the opposite direction, and the formation of one possible complex would be expected to exclude the opposing complex as shown in Figure 7A. The predicted three-dimensional structures shown require DNA bending at the AML/NF-

κB site to form either of the two possible ternary complexes, and this is consistent with the absolute requirement of the AML site for Pro1 transcription in either direction (Figure 1, mutant 5). A potential binding site for an SRY-related protein (AAACAGC; Pontiggia et al., 1994) is located adjacent to the AML-1 site. The AML-1 protein has been implicated in DNA bending in the TCRβ promoter where direct binding of Ets to AML1 augments DNA binding by both proteins and the formation of a ternary DNA-protein complex is facilitated by TCF or SRY DNA bending proteins (Giese et al., 1995). The documented interactions of TBP with C/EBP (Nerlov and Ziff, 1995) and the direct interaction of NF-κB p50 with C/EBPδ (Ruocco et al. 1996) provide evidence to support the direct protein-protein interactions proposed in this model. The three overlapping C/EBP sites at the forward TATA element appear to be involved in "tuning" the probability of forward transcription (see Supplemental Figure S3 and the Supplemental Discussion at http://www.immunity.com/cgi/content/full/21/1/55/DC1).

Proposed Mechanism of Probabilistic *Ly49* Gene Activation

The probabilistic transcriptional switch described in this study provides a mechanism whereby a gene can be transcribed in a subset of cells in an otherwise homogeneous population. However, to generate the irreversible gene activation observed in the *Ly49* gene family, the switch has to be a component of a transcriptional relay system, since the expression of Ly49 proteins in adult NK cells is stable and this would not be the case if Pro1 was active in mature NK cells. The decision made by the Pro1 element in immature NK cells should determine whether the adult promoter (Pro2) will be active in the mature NK cell. Figure 7B shows a model of *Ly49* gene activation that explains the previous observation of inhibitory regions preceding several *Ly49* promoters (Gosselin et al., 2000; McQueen et al., 2001; Saleh et al., 2002), together with the present discovery of stochastic switches in the *Ly49* gene family. The region surrounding exon 1 of the *Ly49* genes would normally exist in a closed chromatin configuration, maintained by a negative element adjacent to the promoter. In the immature NK cell, only Pro1 is active, and each *Ly49* gene will have a characteristic probability of forward or reverse transcription from this element. Forward transcription from Pro1 in the new copy of the gene produced as a result of DNA replication would inhibit the formation of a suppressor complex adjacent to the adult promoter, preventing the generation of the "default" repressed state. When the transcription factors necessary for Pro2 activity are expressed in the mature NK cell, they would initiate transcription from those loci that have been opened by Pro1 forward transcription in the immature NK cell. The reverse Pro1 transcript should simply represent the off state of the switch, since there are no identifiable coding regions 5′ of the Pro1 element. Since the original characterization of Pro1 only analyzed forward transcripts (Saleh et al., 2002), the antisense transcripts produced by several *Ly49* genes were analyzed by RT-PCR of RNA from NK1.1$^+$ bone marrow cells in order to demonstrate reverse Pro1 transcription in immature NK cells. Antisense Pro1 transcripts were found to span the entire *Ly49* intergenic region from the Pro1 element to a consensus polyA addition site preceding the polyA signal of the neighboring gene (see Supplemental Figure S4 at http://www.immunity.com/cgi/content/full/21/1/55/DC1).

The activation of an individual *Ly49* gene should depend on whether or not the Pro1 switch is in the forward (on) or reverse (off) position at the time that the NK cell matures and the transcription factors required for Pro2 activity become available. Each *Ly49* gene therefore contains all the necessary information to generate selective expression that is programmed by the properties of the Pro1 stochastic switch. The Pro1 element is active in the bone marrow (Saleh et al., 2002), and bone marrow stromal cells are required for the activation of *Ly49* genes (Williams et al., 2000), supporting a role for this element in gene activation. A recent study (Tanamachi et al., 2004) has demonstrated the crucial role of the Pro1 element for expression of *Ly49a*. Transgenic mice containing an intact *Ly49a* gene demonstrated variegated expression similar to the endogenous gene, and mice with a transgene lacking the Pro1 element did not express Ly49A.

The identification of a probabilistic binary switch in the *Ly49* gene family has broad implications for the selective activation of genes during development. This type of element provides a simple genetic mechanism for selective activation of genes within a developing tissue. Analysis of the selectively activated KIR gene family of human class I receptors has revealed the presence of Pro1-related elements upstream of the previously characterized promoter, and one of these elements has been shown to be transcriptionally active in vivo (S.K.A., unpublished data). The possible existence of probabilistic switches in the separately evolved human KIR family suggests that this mechanism of selective gene activation may be used by many systems that require programmed control of gene activation. Perhaps other genes that are expressed primarily from only one allele (IL-2, IL-4) are controlled by similar probabilistic gene activation elements. The search for stochastic switches in other systems may provide an explanation for unequal cell fate in many developmental scenarios.

Experimental Procedures

Cell Lines

EL-4 and Jurkat cells were cultured in RPMI 1640 media containing 10% fetal bovine serum. The LNK cell line was cultured in RPMI 1640 containing 50 μM β-mercaptoethanol, nonessential amino acids, 10% fetal bovine serum, HEPES, and IL-2 (8000 IU/ml).

Reporter Constructs

Fragments containing the Pro1 region of *Ly49c, e, g, i,* and *j* were generated by PCR and cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA). Inserts were excised with either SacI/XhoI or XhoI/HindIII and cloned into pGL3 (Promega, Madison, WI) to generate forward and reverse constructs. The two-color reporter vector was generated by inserting a BamHI/SspI fragment from the pd2ECFP vector (Clontech, Palo Alto, CA) containing the destabilized cyan fluorescent protein gene into BglII/Eco47III cleaved pd2EYFP vector (Clontech) containing the destabilized yellow fluorescent protein gene. The *Ly49g* Pro1 core promoter fragments were inserted into the two-color vector using BamHI/XhoI.

Cell Transfection and Luciferase Assays

LNK cells were plated at 3×10^5 cells per well in a 6-well plate and transfected with 1 μg of the individual reporter constructs plus 0.1 μg of the *Renilla* luciferase pRL-SV40 control DNA using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activity of the *Ly49* promoter constructs was normalized relative to the activity of the *Renilla* luciferase produced by the pRL-SV40 control vector. Each construct was tested at least three times, and the results varied by less than 10% between experiments. Constructs were also transfected into EL-4 cells as a negative control, and no activity was found.

Electrophoretic Mobility Shift Assays

EMSA was performed as previously described (Gosselin et al., 2000). The double-stranded oligonucleotide probes used contained the following sequences: Ets/NF-κB, TGTAAATACAAGAACAGGAAAT CTCAAAATAGAGCT; AML-1/NF-κB, AGAGCTGTTTGTGGTTTTCC TAAGATCT; NF-κB-consensus, AGAGCTGTTTGGGATTTTCCTAA GATCT; Rev-TATA+NF-κB, TGCTATGAGTATAAATGAACAGTGTAA ATACAAGAACAGGAAATCTC.

Antibody inhibition of complex formation was performed by incubating the nuclear extract with 1 μg of antibody for 30 min on ice prior to adding the labeled oligonucleotide probe. AML-1, AML-3, NF-κB p50 or p65, C/EBPα, β, and ϵ polyclonal antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant C/EBPγ and δ proteins and polyclonal rabbit antisera were a generous gift from the laboratory of Dr. Peter Johnson, NCI-Frederick. Recombinant TBP was obtained from Santa Cruz, and recombinant NF-κB p50 was from Active Motif (Carlsbad, CA). Anti-TBP antiserum was obtained from Upstate Biotechnology (Lake Placid, NY).

Fluorescence-Activated Cell Sorter Analysis

LNK cells were transfected with two-color vector constructs and selected in G418 (Invitrogen) for 7 days. Cell suspensions were subjected to high-speed cell sorting using a MoFlo (Cytomation, Fort Collins, CO) sorter. YFP (excitation maximum = 513 nm; emission maximum = 527 nm) was detected by 488 nm wavelength argon ion laser (Innova 305C) using a 530/40 band pass filter. CFP (excitation maximum = 433 nm; emission maximum = 475 nm) was detected by krypton ion laser (Innova 302C) tuned to 413 nm wavelength using a 485/25 band pass filter. YFP and/or CFP fractions were collected.

Time-Lapse Fluorescence Microscopy Imaging

LNK cells were grown on 35 mm coverglass bottom dishes (Mattek Corporation, Ashland, MA) and imaged using a Zeiss LSM510 (Carl Zeiss Inc., Jena, Germany) confocal microscope with a 458 nm argon ion laser for CFP excitation with a BP 480–520 emission filter and a 514 nm argon ion laser for YFP excitation with a BP 565–615 emission filter. Large field of view images were acquired using a 20× objective lens with pinholes completely opened for both detectors to give pseudo-conventional images. The resulting images were 12-bit, 1024×1024, with a pixel size of 0.45 μm^2. Contrast and brightness were set using bright samples to ensure no saturation of pixels. Images were acquired every 5 min for 16 hr. Cells were maintained at 37°C and 5% CO_2 in an environmental control chamber stage (20/20 Technology Inc., Wilmington, NC). Differential interference contrast images were acquired to ensure that no focal drift took place and that cells remained viable during the observation period.

Acknowledgments

The authors would like to thank Dr. Peter Johnson for the provision of C/EBP reagents and for helpful discussions. We also acknowledge Drs. Dan McVicar, Lew Mason, Andrew Makrigiannis, Howard Young, and Tom Schneider for critical reading of the manuscript. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Received: February 19, 2004
Revised: May 11, 2004
Accepted: May 18, 2004
Published: July 20, 2004

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